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RESEARCH PAPERS

Racial characterization and genetic diversity of sunflower broomrape populations from Northern Spain

Jebri MALEK[1,3], Lidia del MORAL[1], Juan FERNÁNDEZ-ESCOBAR[2], Begoña PÉREZ-VICH[1] and Leonardo VELASCO[1]

[1] Institute for Sustainable Agriculture (IAS-CSIC), Finca Alameda del Obispo, Avda. Menéndez Pidal s/n, 14004 Córdoba, Spain
[2] Syngenta España S.A., Avenida San Francisco Javier, 24 7ª planta, E-41018 Sevilla, Spain
[3] Present address: Laboratory of Molecular Genetics, Immunology and Biotechnology, Faculty of Science of Tunis, Tunis El Manar, Tunisia

Summary. In Spain, sunflower broomrape (*Orobanche cumana* Wallr.) has been restricted to Cuenca province in Central Spain, and the Guadalquivir Valley in Southern Spain, that represent different gene pools of the species. This pathogenic plant has now spread to other areas such as Castilla y León region in Northern Spain. The racial status and genetic diversity were investigated in six populations of sunflower broomrape collected in several provinces of Castilla y León. Evaluation of virulence to a set of differential host genotypes classified three of the populations as race F, while the other three populations were classified as a race below F, probably race E. Genetic diversity analysis using a set of 20 SSR markers showed that the broomrape populations from new areas of Northern Spain are mainly derived from the Guadalquivir Valley gene pool. Introgression from the Cuenca gene pool was observed in one of the populations, in which the percentage of polymorphic loci was 31%, Shanon´s diversity index was 0.17, and the average number of pairwise differences was 1.69, compared to zero for the three parameters in the other five populations. The absence of race F individuals in the populations classified as race below F indicated that seed migration took place, probably before the generalized expansion of race F in the Guadalquivir Valley area, at the beginning of the 1990s.

Key words: Gene flow, *Orobanche cumana*, parasitic weed, SSR markers, virulence.

Introduction

Sunflower broomrape (*Orobanche cumana* Wallr.) is native to a region across South-eastern Europe and Central Asia, where it is found in the wild parasitizing Compositae species, mainly of the genus *Artemisia* (Beck-Mannagetta, 1930). Although Beck-Mannagetta (1930) considered *O. cumana* to be a variety of *O. cernua* Loefl., there is now broad consensus that *O. cumana* is a separate species (Fernández-Martínez *et al.*, 2015). Sunflower parasitization by *O. cumana* was first recorded at the end of the 19th century, when it was detected in Russia (Škoriç, 2012). Broomrape is now distributed as a damaging parasitic weed of sunflower, mainly throughout the host's natural distribution area, i.e. the Black Sea and Caspian Sea regions (Kaya, 2014). Nevertheless, sunflower broomrape has extended to other areas far from its natural distribution region, such as Spain. There, broomrape was first found in the central region, mainly in Cuenca province, and later in the south, mainly in the Guadalquivir Valley (Alonso *et al.*, 1996). Pineda-Martos *et al.* (2013) and Molinero-Ruiz *et al.* (2014) found that *O. cumana* populations from these Spanish regions are genetically distant, probably corresponding to two separate introductions (Pineda-Martos *et al.*, 2013).

For many years in Spain, sunflower broomrape has been confined to Cuenca Province and the Guadalquivir Valley. The occurrence of the pathogenic plant in sunflower crops in other areas was not re-

Corresponding author: L. Velasco
E-mail: lvelasco@ias.csic.es

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ported until 2008 in Castilla y León region in the North of Spain (Fernández-Escobar *et al*., 2009). Pineda-Martos *et al.* (2013) analysed one population from this region (Valladolid province) using microsatellite markers, and concluded that it was genetically very close to the gene pool of the Guadalquivir Valley. Since Castilla y León contains close to one third of the area of sunflower crops in Spain, and that broomrape infestation is expanding through the region in recent years, it is important that the racial status and genetic diversity of the populations are established. These are the objectives of the research reported here.

Materials and methods

Orobanche cumana populations

This study included 14 *O. cumana* populations, six from Castilla y León region, four from the Guadalquivir Valley, and four from Cuenca Province (Table 1). The populations from the Guadalquivir Valley and Cuenca Province were selected to represent the *O. cumana* gene pools previously identified in both areas (Pineda-Martos *et al*., 2013). They were included in the study to compare the populations of the Castilla y León region with those of the gene pools previously identified in Spain. Figure 1 shows the location of the 14 populations used in the study.

Race classification

Seven differential inbred sunflower lines and hybrids were used for race classification of the sunflower broomrape populations collected in Northern Spain. These were: line B117, which is susceptible to all races (Pineda-Martos *et al*., 2014b), line NR5 and hybrid Sanbro, both resistant to race E, and line P96 and commercial hybrids Kiara, Transol, and ES-Artic, which are resistant to races F from the Guadalquivir Valley (F_{GV}) and Cuenca (F_{CU}). Broomrape population SP, previously characterized as race F_{GV} and highly virulent on sunflower lines B117 and NR5 (Martín-Sanz *et al*., 2016), was used as a control, to check the level of parasitization on susceptible lines. Differential lines for races below E were not included since the only lines available, developed in Romania (Vrânceanu *et al*., 1980), have been found to be ineffective for accurate race classification of Spanish broomrape populations (Molinero-Ruiz and Domínguez, 2014).

Table 1. *Orobanche cumana* populations used in this study.

Population	Collection site (region, province, county)	Collection year, Reference
IN-155	Castilla y León, Valladolid, Medina del Campo	2008
IN-194	Castilla y León, Salamanca, Aldeaseca de la Frontera	2011
IN-205	Castilla y León, Zamora, Vadillo de la Guareña	2012
IN-224	Castilla y León, Soria, Nepas	2014
IN-225	Castilla y León, Segovia, Cantalejo	2014
IN-226	Castilla y León, Burgos, Sotresgudo	2014
CU-05	Castilla La Mancha, Cuenca, La Almarcha	1996, Pineda-Martos *et al*., 2013
CU-07	Castilla La Mancha, Cuenca, Carrascosa del Campo	1996, Pineda-Martos *et al*., 2013
CU-12	Castilla La Mancha, Cuenca, Palomares del Campo	2008, Pineda-Martos *et al*., 2013
IASCum-4	Castilla La Mancha, Cuenca, Villarejo de Fuentes	2008, Pineda-Martos *et al*., 2014a
CO-02	Andalucía, Córdoba, Aldea Quintana	2008, Pineda-Martos *et al*., 2013
SE-10	Andalucía, Sevilla, Écija	2007, Pineda-Martos *et al*., 2013
EK-23	Andalucía, Córdoba, Córdoba	1995, Rodríguez-Ojeda *et al*., 2013
SP	Andalucía, Sevilla, Écija	2001, Martín-Sanz *et al*., 2016

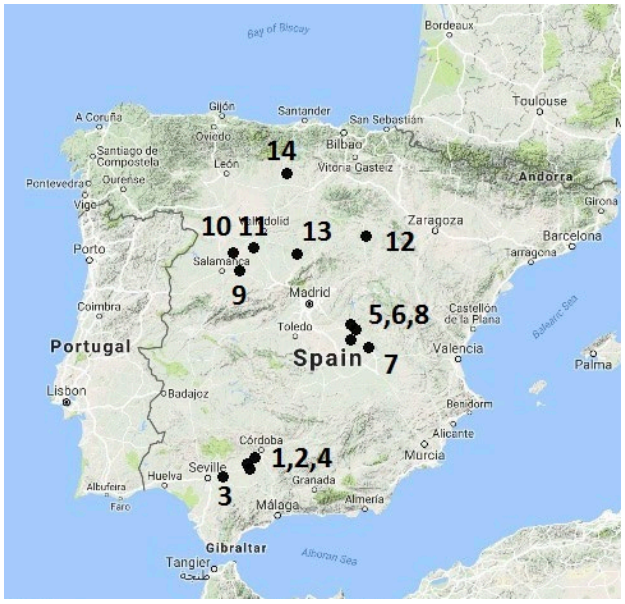

Figure 1. Locations where the *Orobanche cumana* populations were collected. **1** = CO-02; **2** = SE-10; **3** = EK-23; **4** = SP; **5** = IASCum-4; **6** = CU-12; **7** = CU-05; **8** = CU-07; **9** = IN-155; **10** = IN-194; **11** = 205; **12** = IN-224; **13** = IN=225; **14** = IN-226.

Sunflower plants of the differential lines were grown in pots containing soil inoculated artificially with seeds of the different broomrape populations. For each pot (7 × 7 × 8 cm, filled with a mixture of sand and peat (1:1 v:v), 25 mg of broomrape seeds were added, and the inoculated substrate was shaken in a plastic bag to obtain homogeneous distribution of the seeds. Seeds of the sunflower lines and hybrids were germinated on moistened filter paper in Petri dishes and then planted in the pots containing the inoculated substrate. Eight pots for each combination of sunflower line and broomrape population were used. The plants were initially grown for 3 weeks in a growth chamber at 25ºC/20ºC (day/night) with a 16h photoperiod, after which they were transplanted to pots containing 3 L of uninfested sand-silt-peat (2:1:1 v:v:v) substrate, and maintained under open air conditions. The plants were watered daily and not fertilized. The numbers of *O. cumana* shoots per sunflower plant were counted at sunflower maturity.

Tissue collection, DNA extraction and SSR analysis

Twenty broomrape shoots per population were collected before flowering on plants of the susceptible line B117 from the race classification study, and immediately frozen at -80ºC. The plant tissues were then lyophilized and ground to a fine powder using a laboratory ball mill. DNA was extracted from individual plants using the protocol described by Rogers and Bendich (1985) with small modifications, which included (i) addition of 0.1% (w:v) ascorbic acid, 0.1% (w:v) diethyldithiocarbamic acid sodium salt, and 0.2% (v:v) 2-mercaptoethanol to the CTAB extraction buffer just before use; (ii) increase of the CTAB buffer incubation time to 30 min; and (iii) use of chloroform instead of chloroform:isoamyl alcohol. Equal amounts of DNA of 20 broomrape plants from each population were pooled and used as a template for PCR amplification for assessment of interpopulation diversity. For assessment of intrapopulation diversity, DNA from 12 individual plants was used as a template for PCR amplification. This number of plants per population was based in a previous study of Pineda-Martos *et al.* (2013) on populations from Spain, in which it was found sufficient to indicate the existence of intrapopulation diversity. Microsatellite analyses were carried out as described in Pineda-Martos *et al.* (2014a), using 20 high-quality, polymorphic SSR primer pairs reported in that work. These were: Ocum-003, Ocum-023, Ocum-052, Ocum-059, Ocum-063, Ocum-070, Ocum-074, Ocum-075, Ocum-081, Ocum-087, Ocum-091, Ocum-092, Ocum-108, Ocum-122, Ocum-141, Ocum-151, Ocum-160, Ocum-174, Ocum-196, and Ocum-197. Amplification products were separated by gel electrophoresis using 3% Metaphor agarose (BMA) in 1× TBE buffer and SaveView Nucleic Acid Stain (NBS Biologicals Ltd), and then viewed under UV light. A 100 bp DNA ladder (Solis BioDyne) was used as a standard molecular weight marker. Gel bands were scored manually with the aid of Quantity One® 1-D Analysis Software (Bio-Rad Laboratories Inc.). Amplified fragments were scored for the presence (1) or absence (0) of homologous bands and compiled into a binary data matrix, or for their estimated molecular weight and compiled into a codominant data matrix.

Analysis of inter-population diversity

Cluster analysis was conducted using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) from a dissimilarity matrix built with DICE dissimilarity index, using NTSYSpc ver. 2.21q (Applied Biostatistics Inc.). The goodness of fit of

the resulting dendrogram to the dissimilarity matrix was calculated using the cophenetic correlation coefficient.

Analysis of intra-population diversity

The percentage of polymorphic loci (P) and Shannon's diversity index (I) were calculated for all loci in each population using GenAlEx ver. 6.5 (Peakall and Smouse, 2012). The average number of pairwise differences between individuals within each population was calculated using Arlequin ver. 3.5.2.2 (L. Excoffier, CMPG, University of Bern, Switzerland).

Results

Race classification of the sunflower broomrape populations

Three of the broomrape populations collected in Northern Spain (IN-155, IN-224 and IN-226) were classified as race F because they parasitized sunflower plants of the race-E resistant genotypes NR5 and Sanbro, and did not parasitized race-F resistant genotypes P-96, Kiara, Transol or ES-Artic. In the case of population IN-226, the number of broomrape shoots per sunflower plant was considerably less than in the other three race F populations. This indicates a lower proportion of individuals with ability to overcome *Or5* resistance than in the other populations. Conversely, the other three populations (IN-194, IN-205 and IN-225) did not grow on NR5 and Sanbro, so they were classified as race E or below E (Table 2).

Interpopulation relatedness

Cluster analysis on bulked DNA of the populations revealed that five (IN-155, IN-194, IN-205, IN-224, and IN-226) showed similar genetic patterns to the populations of the Guadalquivir Valley, whereas one of population (IN-225) clustered together with the populations of the Guadalquivir Valley, but at some separation (Figure 2).

Intrapopulation diversity

The five populations from Northern Spain grouped together with the Guadalquivir Valley populations showed no intrapopulation diversity, with all the individuals showing the same allelic pattern for the SSR markers. Conversely, population IN-225 showed some intrapopulation diversity (Figure 3). For population IN-225, the percentage of polymorphic loci was 31.25%, Shanon´s diversity index was 0.17, and the average number of pairwise differences

Table 2. Percentage of susceptible plants (%S) and average number of sunflower broomrape shoots in susceptible plants (Xs) in seven sunflower differential lines and hybrids inoculated with six broomrape populations collected in northern Spain and race-F population SP used as a control.

Line/Hybrid[a]	Broomrape population													
	IN-155		IN-194		IN-205		IN-224		IN-225		IN-226		SP	
	%S	Xs	%S	Xs	%S	Xs	%S	Xs	%S	Xs	%S	Xs	%S	Xs
B117	100	39	100	31	100	21	100	34	100	33	100	20	100	27
NR5	100	29	0	-	0	-	100	30	0	-	100	4	100	28
Sanbro	100	31	0	-	0	-	100	35	0	-	100	7	100	36
P96	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Kiara	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Transol	0	-	0	-	0	-	0	-	0	-	0	-	0	-
ES-Artic	0	-	0	-	0	-	0	-	0	-	0	-	0	-

[a] Line B117 is susceptible to all broomrape races; line NR5 and hybrid Sanbro are resistant to race E; Line P96 and hybrids Kiara, Transol, and ES-Artic are resistant to races F of the Guadalquivir Valley (F_{GV}) and Cuenca (F_{CU}).

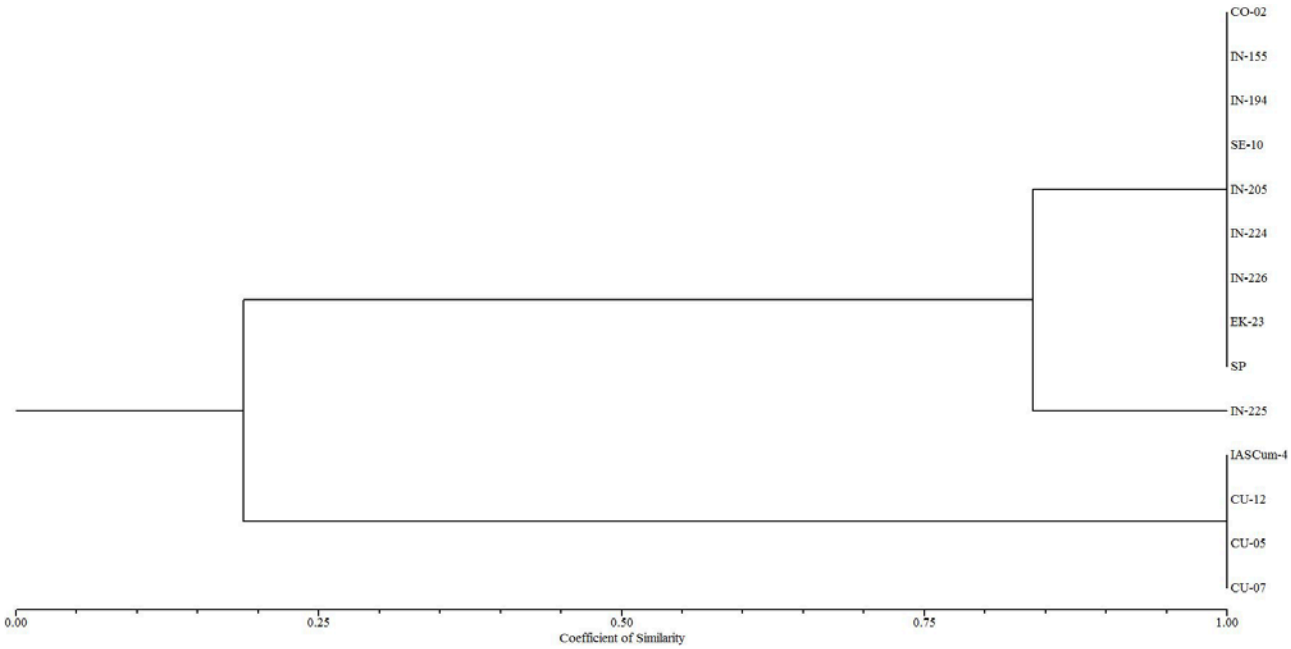

Figure 2. Tree plot of six populations collected in northern Spain (IN-155, IN-194, IN-205, IN-224, IN-225, IN-226) and reference populations from the Guadalquivir Valley (CO-02, SE-10, EK-23, SP) and Cuenca province (IASCum-4, CU-05, CU-07, CU-12).

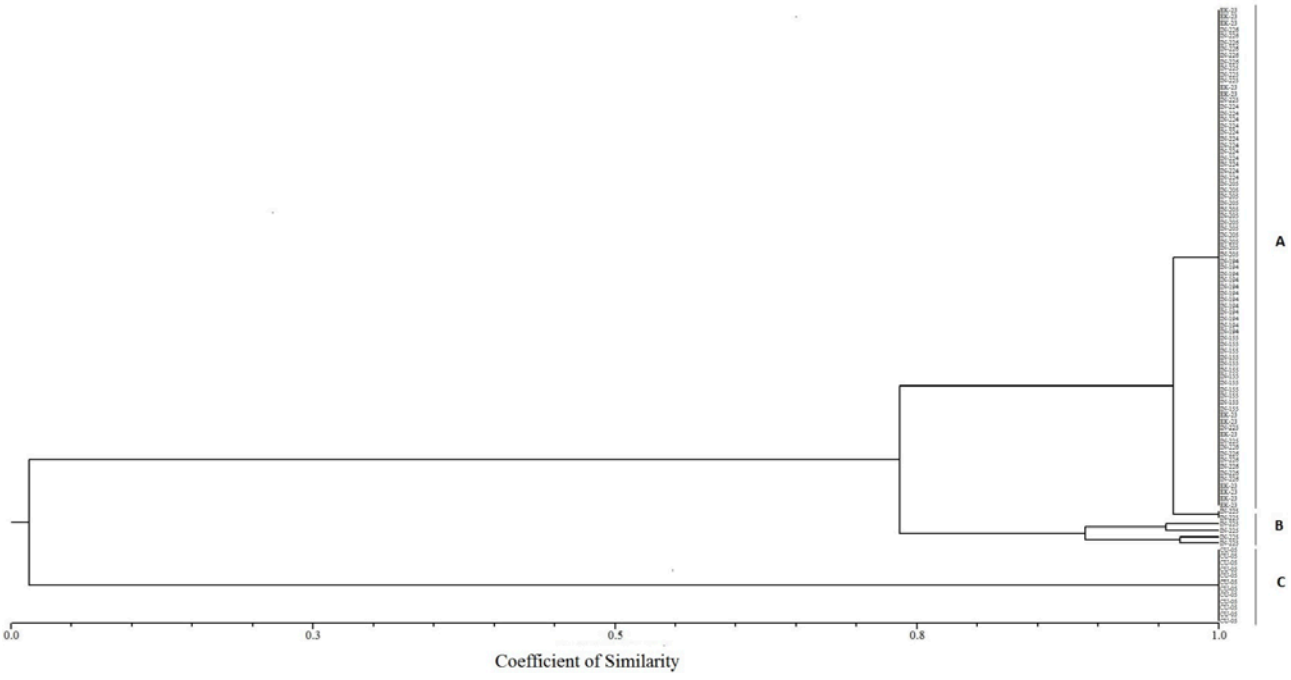

Figure 3. Tree plot of 96 individual sunflower broomrape plants from six populations collected in northern Spain (IN-155, IN-194, IN-205, IN-224, IN-225 and IN-226), and two reference populations from the Guadalquivir Valley (EK-23) and Cuenca province (CU-05). The plot includes 12 individuals per population. The three clusters correspond to the homogeneous groups of the Guadalquivir Valley (A) and Cuenca (C), and a group of six individuals from population IN-225 (B).

within the population was 1.69. These values were zero for all three parameters in the other populations. For all the polymorphic markers, the alleles present in population IN-225 were those of the Guadalquivir Valley and Cuenca province.

Discussion

Sunflower broomrape is native from a region between Eastern Europe and Central Asia, where populations parasitizing wild flora and sunflower crops co-exist (Pineda-Martos *et al.*, 2014b). In Spain, the species is not found in the wild, but occurs exclusively parasitizing sunflower crops (Pujadas-Salvà and Velasco, 2000). Pineda-Martos *et al.* (2013) identified two contrasting genetic pools of sunflower broomrape in Spain, one in Cuenca province in Central Spain, and the other in the Guadalquivir Valley in Southern Spain. Populations from other areas evaluated in their research, corresponding to recent expansion of the parasite, mainly belonged to one of the gene pools. However, some of the populations were identified as mixtures of both genetic pools. That study included only one population from Northern Spain, which was grouped in the Guadalquivir Valley gene pool. The present research confirmed the close relatedness between populations from Northern Spain with the gene pool of the Guadalquivir Valley, with five out of six populations showing no genetic differences and one population showing a main genetic background of the Guadalquivir Valley pool, with putative introgressions of the Cuenca gene pool. Such introgressions were also observed by Pineda-Martos *et al.* (2013), and more recently by Martin-Sanz *et al.* (2016), who suggested that populations with increased virulence observed in the Guadalquivir Valley (race G_{GV}) were the result of genetic recombinations between individuals of both Spanish gene pools.

It is of particularly note that three of the populations from Northern Spain were classified as race below F (E or below E) and the other three populations were classified as race F. More detailed racial classification in populations below F could not be conducted due to the lack of adequate differential host lines, since those developed in Romania in the late 1970s (Vrânceanu *et al.*, 1980) are not fully valid for differentiating Spanish populations (Molinero-Ruiz and Domínguez, 2014). The populations below F contained no race-F individuals, since no broomrape shoot developed from the two race-E differential genotypes used in the evaluation. Molinero-Ruiz *et al.* (2008) evaluated 12 populations collected from 1988 to 1999 in the Guadalquivir Valley, and found that they contained varying proportions of race-F individuals. Accordingly, populations of the Guadalquivir Valley gene pool containing no race-F individuals should correspond with seed movements that took place before the generalized expansion of race F in the Guadalquivir Valley area at the beginning of the 1990s (Alonso *et al.*, 1996). However, sunflower broomrape was not detected in the area until 2008 (Fernández-Escobar *et al.*, 2009). This cannot be satisfactorily explained with the available information. If this hypothesis is correct, the reason for parasitization not being detected until very recently should be investigated. This may be due to adaptation of the populations to the new environments.

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